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Original Article

Involvement of leukotriene B_4 in spontaneous itch-related behaviour in NC mice with atopic dermatitis-like skin lesions

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Abstract: To elucidate the mechanisms of severe itch in atopic dermatitis, we investigated the role of leukotriene B_4, a potent itch mediator, in spontaneous itch-related behaviour in NC mice with atopic dermatitis-like skin lesions. Topical application of the BLT leukotriene B_4 receptor antagonist ONO-4057 inhibited spontaneous itch-related behaviour. The concentration of leukotriene B_4 was significantly increased in the lesional skin. The expression levels of 5-lipoxygenase were also elevated in the lesional skin, yet present throughout the epidermis of both healthy and lesional skin. These results suggest a role for leukotriene B_4 in chronic dermatitis-related itch. Sphingosylphosphorylcholine (SPC) was increased in the epidermis of the lesional skin. Moreover, intradermal injection of SPC elicited itch-related behaviours in healthy mice. Because SPC induces itch-related responses through the production of leukotriene B_4 in keratinocytes (*J Invest Dermatol*, 129, 2009, 2854), these results suggest that an increase in SPC induces leukotriene B_4-mediated itching in chronic dermatitis. BLT1 receptor and 5-lipoxygenase in the skin may be effective pharmacological targets for the treatment of itch in atopic dermatitis.

Key words: atopic dermatitis – itch – leukotriene B_4 – scratching – sphingosylphosphorylcholine

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Introduction

Atopic dermatitis is a chronic inflammatory skin disease, and itch is its major diagnostic criterion (1). The hyper-reactivity of the immune response is not present in all patients with atopic dermatitis (2), and itch is usually resistant to H_1 histamine receptor antagonists in these patients (3). Epidermal barrier dysfunction is a key event in the development of atopic dermatitis (4), and scratching of the itching skin worsens dermatitis and itch (5). Detailed understanding of the mechanisms and mediators of itch in atopic dermatitis is limited. Leukotriene B_4 (LTB_4) is a metabolite of arachidonic acid produced by the actions of 5-lipoxygenase and LTA_4 hydrolase (6). LTB_4 is increased in the lesional skin of patients with atopic dermatitis (7,8). Azelastine, an H_1 histamine receptor antagonist that inhibits the action and production of LTB_4 (9), suppresses itch in atopic dermatitis patients (10). In animal models of itch, LTB_4 elicits scratching, an itch-related response (11), and is involved in itch-related responses to intradermal injections of substance P (12) and nociceptin (13), and allergies such as passive cutaneous anaphylaxis and contact dermatitis (14,15). Thus, the main aim of this study was to determine whether LTB_4 is involved in atopic dermatitis-related itch.

NC mice develop chronic dermatitis, hyperplasia, dry skin and spontaneous itch-related behaviours when kept for a long time in a conventional environment (16,17). Serum IgE is markedly increased in NC mice with chronic dermatitis, but it is not key for spontaneous itch-related behaviours (17). These features are similar to those of patients with atopic dermatitis. The role of LTB_4 in itching in chronic dermatitis is unknown, although recent studies have revealed the involvement of some endogenous factors and receptors such as interleukin-31 and proteinase-activated receptor 2 (18–20). In this study, therefore, we investigated whether LTB_4 is involved in spontaneous itching in NC mice with chronic dermatitis.

In the lesional stratum corneum of patients with atopic dermatitis, there is a reciprocal relationship between decrease in ceramide and increase in sphingosylphosphorylcholine (SPC) (21). The decrease in ceramide – probably due to an increase in the conversion of sphingomyelin to SPC (22) – may be a cause for cutaneous barrier disruption and dry skin (23). Recently, it has been shown that intradermal injection of SPC induces itch-related responses in mice (24,25) and that LTB_4 is involved in the action of SPC (25). Therefore, this study also examined whether SPC is increased in the skin of NC mice with chronic dermatitis.

Materials and methods

Animals

NC/Jic mice were bred in a specific pathogen-free environment at the Division of Animal Resources and Development, Life Science Research Center, University of Toyama. They were kept under controlled temperature (23 ± 1°C), humidity (60% ± 5%) and light (light on 8:00–20:00 h). Food and water were freely available. All mice except healthy controls were transferred to a conventional environment at 4–5 weeks of age. To increase the incidence of chronic dermatitis, they were kept together with mite-infected mice with chronic dermatitis for 2 weeks (17). They were used for experiments at 13–25 weeks of age; age-matched healthy mice were used as control in each series of experiments. All procedures for animal experiments were approved by the Committee for Animal Experiment at the University of Toyama and were in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Materials

ONO-4057, 5-[2-(2-carboxyethyl)-3-[6-(4-methoxyphenyl)-5*E*-hexenyl] oxyphenoxy] valeric acid, was dissolved in 100% ethanol (Wako Pure Chemical Ind., Osaka, Japan) and was applied to the rostral back of each mouse in a volume of 100 μl 1 h before the observation. SPC (Sigma, St. Louis, MO, USA) was dissolved in physiological saline containing 1% ethanol and injected intradermally into the rostral back of each mouse in a volume of 50 μl.

Behavioural experiments

The animals were placed individually in an acrylic cage composed of four equal-sized cells ($13 \times 9 \times 40$ cm) for at least 1 h for acclimation. Then, their behaviours were videotaped for 1 h with personnel kept out of the observation room. Spontaneous scratching toward any rostral regions of the body by the hind paws was counted (17). In experiments examining the effects of topical application or intradermal injection of agents, the hair was clipped from the rostral part of the back the day before the experiment and spontaneous scratching toward the hair-clipped region was counted. As mice make several rapid scratch movements for periods of about 1 s, a series of these movements was counted as one bout of scratching (26).

Enzyme immunoassay

The skin (1.7 cm in diameter) of the rostral back was removed, immediately shredded with scissors and put into 2 ml of ice-chilled ethanol containing 10 μM indomethacin and 10 μM zileuton to inhibit cyclooxygenase and 5-lipoxygenase, respectively. After being homogenized with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland), the sample was centrifuged at $600 \times g$ at 4°C for 5 min. The supernatant (1 ml) was mixed with 5 ml of double-distilled water, and pH was adjusted to 3.5 with HCl. The sample was then applied to a C18 Sep-Pak cartridge (Waters, Milford, MA, USA) equilibrated with methanol. After the cartridges were washed with hexane followed by double-distilled water, lipids were eluted with ethanol. After evaporation of the eluate, the residue was suspended in an enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI, USA) for the assay of LTB_4. The protein concentration of the residue was determined by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The LTB_4 content in the skin was normalized to protein amount.

Western blotting

The skin of the rostral back was removed, and proteins were extracted with a lysis buffer (20 mM Tris–HCl [pH 7.5], 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM phenylmethyl sulphonyl fluoride, 10 μg/ml aprotinin and 1 μg/ml leupeptin). Proteins were separated by electrophoresis using a sodium dodecyl sulphate–polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk solution for 1 h, the membrane was reacted with goat anti-5-lipoxygenase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or mouse anti-β-actin monoclonal antibodies (Sigma) at 4°C overnight. Subsequently, it was incubated with horseradish peroxidase-conjugated anti-goat IgG (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA) or horseradish peroxidase-conjugated anti-mouse IgG antibodies (GE Healthcare Bio-Sciences Co.) for 1 h at room temperature and then treated with chemiluminescence reagents (GE Healthcare Bio-Sciences Co.). Chemiluminescent signals were detected using an X-ray film and analysed using the NIH Image program (National Institutes of Health, Bethesda, MD, USA). Signal intensity was normalized to that of β-actin.

Immunohistochemistry

The skin of the rostral back was removed, fixed with 10% formalin, embedded in paraffin and cut with a microtome into 3-μm thick sections. After deparaffinization, the sections were treated with methanol containing 0.3% hydrogen peroxide and then with 0.3% Triton X-100. After treatment with 0.25% fetal bovine serum to block immunoglobulin binding, the sections were incubated with goat anti-5-lipoxygenase antibody (Santa Cruz Biotechnology Inc.) at 4°C overnight, followed by biotin-conjugated rabbit anti-goat IgG antibody (Dako, Carpinteria, CA, USA) and then horseradish peroxidase-conjugated avidin (Invitrogen, Carlsbad, CA, USA). The sections were subsequently treated with 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Ind.) and counterstained with haematoxylin. The staining was observed using a light microscope (AX-80; Olympus, Osaka, Japan) coupled to a CCD camera (Axio Cam; Carl Zeiss, Jena, Germany).

Thin-layer chromatography (TLC)

After washing of the skin in distilled water at 60°C for 30 s, epidermal sheets were exfoliated from the skin and dried up. The sheet (1 mg) was treated with 100 μl of a mixture of chloroform/methanol (2:1) at 4°C overnight. The supernatant was spotted on a TLC silica gel plate (60 F_{254}; Merck KGaA, Darmstadt, Germany), and lipid species were separated and identified using a solvent mixture of chloroform/methanol/acetic acid/water (50:30:8:5) and an authentic sample of SPC. The plate was treated with ethanol containing 5% phosphomolybdic acid (Wako) and heated on a hot plate (100°C) to visualize lipids. The density of a spot of SPC was analysed using the NIH Image program (National Institutes of Health).

Data processing

Data are presented as means ± standard error of the mean (SEM). Statistical significance was analysed using Dunnett's multiple comparisons or Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

The effect of BLT LTB_4 receptor antagonist on spontaneous scratching in mice with dermatitis

Figure 1a shows the number of spontaneous scratch bouts of individual mice toward the rostral part of the body by the hind paws. Mice with chronic dermatitis scratched more frequently than age-matched healthy mice. Although most of the mice with chronic dermatitis scratched more frequently than age-matched healthy mice, some of them showed less frequent scratching than their healthy counterparts. Therefore, we observed spontaneous scratching a couple of days before the start of the next experiment, and in the subsequent experiments, we excluded mice showing less frequent scratching under baseline conditions.

Figure 2 shows the number of spontaneous scratch bouts toward the hair-clipped region of application of the drug. Topical application of the BLT LTB_4 receptor antagonist ONO-4057 at 0.1% or 1% produced a dose-dependent inhibition of the spontaneous scratching. The effect was statistically significant at the concentration of 1% (Fig. 1b).

LTB_4 in the skin

The levels of LTB_4 were significantly increased in the lesional skin of mice with chronic dermatitis, as compared with the skin of healthy mice (Fig. 2).

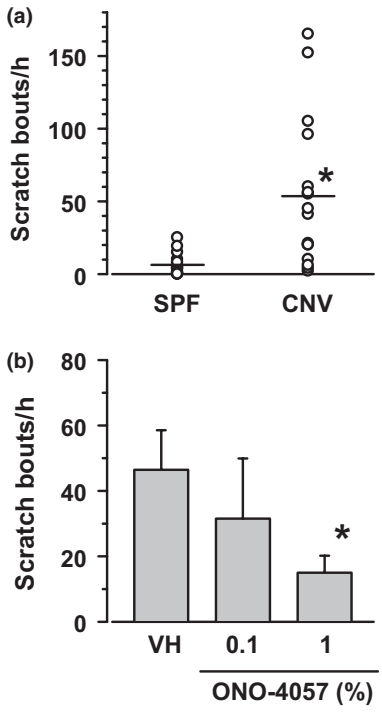

Figure 1. Effects of BLT leukotriene B_4 receptor antagonist on spontaneous scratching in NC mice with chronic dermatitis. (a) Comparison of spontaneous scratching between healthy and dermatitis mice. Spontaneous scratching toward the rostral part of the body by the hind paws was counted for 1 h at 13 weeks of age. Healthy mice were kept under specific pathogen-free (SPF) conditions, and dermatitis mice were transferred to a conventional environment (CNV) at 4–5 weeks of age. Open circles represent the values of individual animals; the horizontal bars represent the means (n = 16 each). *P < 0.05 (Student's t-test). (b) Effects of the BLT receptor antagonist ONO-4057. ONO-4057 or the vehicle (VH) was applied to the rostral back skin of the mouse 1 h before the observation. Scratching toward the application site by the hind paws was counted. Values represent the mean ± SEM of seven to eight animals. *P < 0.05, as compared with VH (Dunnett's multiple comparisons).

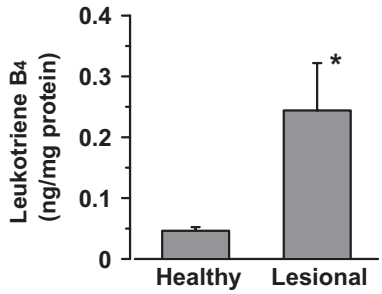

Figure 2. Leukotriene B_4 in the skin of NC mice. The content of leukotriene B_4 in the rostral back skin of healthy or dermatitis mice was measured by enzyme immunoassay. Values represent the mean ± SEM of 20 animals. *P < 0.05 (Student's t-test).

Distribution of 5-lipoxygenase in the skin

5-Lipoxygenase was detected by immunohistochemistry throughout the epidermis and scattered in the dermis of healthy skin (Fig. 3a). Epidermal hyperplasia was markedly observed in lesional skin, where 5-lipoxygenase was also present throughout the epidermis (Fig. 3a). After normalization, we found that the expression levels of 5-lipoxygenase were significantly increased in the lesional skin, as compared with the healthy skin (Fig. 3b).

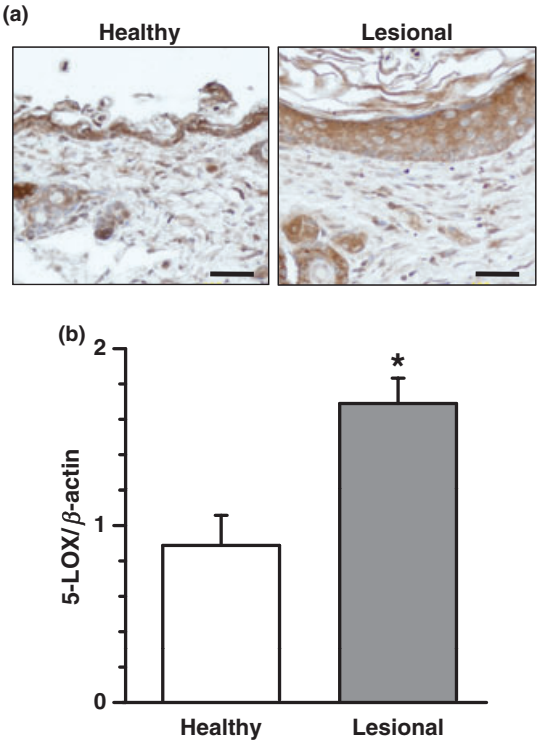

Figure 3. Localization and expression levels of 5-lipoxygenase (5-LOX) in the skin of NC mice. (a) Immunohistochemical staining of 5-LOX in sections of the rostral back skin of healthy or dermatitis mice. Scale bars represent 100 μm. (b) Expression levels of 5-LOX in the skin. 5-LOX was determined by western blotting and normalized to the expression levels of β-actin. Values represent the mean ± SEM of three animals. *P < 0.05 (Student's t-test).

Expression of SPC in the skin and relation with the scratching behaviour

Thin-layer chromatography analysis revealed a detectable amount of SPC in the epidermis of healthy mice. Moreover, the levels of SPC were significantly higher in the epidermis of lesional skin than in healthy skin (Fig. 4a). An intradermal injection of SPC (100 nmol/site) significantly elicited scratching in the healthy mice (Fig. 4b).

Discussion

One important finding in the present study is that topical application of a BLT LTB_4 receptor antagonist inhibited spontaneous scratching of NC mice with chronic dermatitis, suggesting that LTB_4 and its receptor are involved in spontaneous itching in chronic dermatitis. This suggestion is supported by the result that LTB_4 concentration was markedly increased in the lesional skin. LTB_4 is a potent endogenous itch mediator (11) and has been shown to be involved in acute itching induced by intradermal injections of substance P, nociceptin and SPC (12,13,25), and allergy (14,15) in ICR mice. This is the first report of the involvement of LTB_4 in itching in atopic dermatitis-like skin lesions.

Leukotriene B_4 is derived from arachidonic acid via the actions of 5-lipoxygenase and LTA_4 hydrolase (6). The present study showed the presence of 5-lipoxygenase throughout the epidermis of NC mice, suggesting its localization in epidermal keratinocytes,

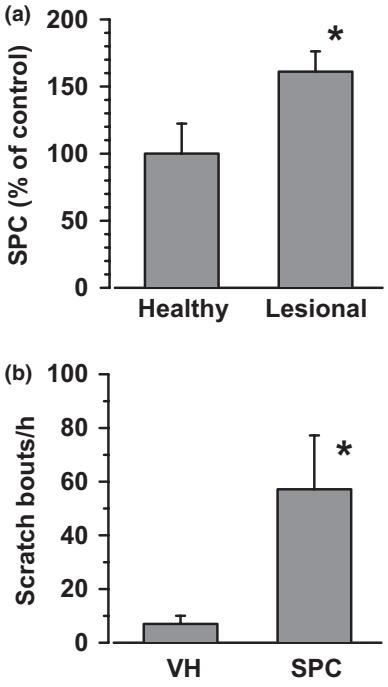

Figure 4. Sphingosylphosphorylcholine (SPC) in the skin and SPC-induced scratching in NC mice. (a) The content of SPC was measured by thin-layer chromatography. An averaged SPC content in healthy mice served as control (%). Values represent the mean ± SEM of six animals. $*P < 0.05$ (Student's *t*-test). (b) SPC (100 nmol/site) or vehicle (VH: saline containing 1% ethanol) was injected intradermally into the rostral back skin of healthy mice. Values represent the mean ± SEM of eight animals. $*P < 0.05$ (Student's *t*-test).

which have also been shown to express LTA_4 hydrolase (27). The skin lesions showed marked keratinocyte hyperplasia (acanthosis) and an increase in 5-lipoxygenase-expressing keratinocytes. An increase in the normalized levels of 5-lipoxygenase in the skin suggests that the amount of this enzyme in each keratinocyte was also increased. This may be a cause for the increased concentration of cutaneous LTB_4. The mechanisms underlying 5-lipoxygenase-increased expression are still not clear. The transcription factor SP1 plays an important role in the regulation of the expression of 5-lipoxygenase (28) and is increased in the inflamed skin (29). Thus, it would be interesting to examine changes in SP1 in the lesional skin of NC mice with chronic dermatitis. We do not rule out the possibility that LTB_4 might also be produced by Langerhans cells, mast cells and macrophages – cells that have previously been shown to express 5-lipoxygenase (30) and LTA_4 hydrolase (31–33).

The amount of ceramide is decreased in the stratum corneum of atopic dermatitis patients and leads to dryness and impaired barrier function of the skin (21,23). In the healthy skin, ceramide is synthesized from sphingomyelin by sphingomyelinase in the stratum corneum (34). In the lesional skin of atopic dermatitis patients, sphingomyelin deacylase activity is increased. Sphingomyelin is thus metabolized to SPC, which leads to a decrease in ceramide (21,22,34). In the present study, SPC was found to be increased in the lesional skin of NC mice. An intradermal injection of SPC elicits scratching in healthy mice (24,25, present experiment) through the production of LTB_4 in keratinocytes (and direct action on primary afferents) (25). Therefore, it is suggested that an increase in SPC is also responsible for LTB_4-mediated spontaneous itch in NC mice with chronic dermatitis.

Substance P is increased in the skin of atopic dermatitis patients and NC mice with chronic dermatitis (35,36). LTB_4 is involved in itching induced by intradermal injection of substance P (12). Thus, it is also possible that an increase in substance P in lesional skin is involved in LTB_4-mediated itching in chronic dermatitis.

There are two LTB_4 receptor subtypes, the BLT1 and BLT2 receptors, which have high and low binding affinities for LTB_4, respectively, and both are G-protein-coupled receptors (37,38). The BLT antagonist ONO-4057 blocks both BLT1 and BLT2 receptors (37–39). BLT1 mRNA is expressed predominantly in leucocytes in human and mice (39). BLT2 mRNA is expressed in many tissues, such as spleen and leucocytes, in human but not mice (39). Our previous report has shown that BLT1, but not BLT2, is expressed in the dorsal root ganglia and skin of mice (40). BLT1 protein is also expressed in primary afferent and keratinocytes (40). Taken together, it is suggested that LTB_4 produced in chronic dermatitis skin elicits itch through BLT1 receptors on primary afferents and keratinocytes. Most BLT1 receptor-positive neurons (about 80%) are also positive for transient receptor potential vanilloid 1 (TRPV1) receptor (40). TRPV1-positive sensory neurons may be involved in itch-related signalling (41,42). Thus, LTB_4 produced in lesional skin may act on the BLT1 receptors on TRPV1-positive primary afferents. Keratinocytes also express BLT1 receptors (40), and LTB_4 acts on epidermal keratinocytes (43). Stimulation of the BLT1 receptor results in the increase in intracellular Ca^{2+} ions (37,40), which in turn activate phospholipase A_2 and arachidonic acid synthesis (44). Therefore, it is suggested that the autocrine-like action of LTB_4 in keratinocytes produces pruritogenic arachidonic acid metabolites, such as LTB_4 itself and thromboxane A_2, and other itch mediators and enhancers, including nitric oxide, nociceptin and SPC (13,25,45,46).

Azelastine, an H_1 histamine receptor antagonist that inhibits the action and production of LTB_4 (9), suppresses nocturnal scratching in patients with atopic dermatitis after 3 days of administration (10). Subjective scores for pruritus have been reported to be decreased in five of six patients with atopic dermatitis after 6 weeks of zileuton administration (47). Zileuton is a 5-lipoxygenase inhibitor and inhibits LTB_4 production in the skin after intradermal injections of SPC and substance P, endogenous pruritogens (12,22). Thus, the present results suggest that the antipruritic effects of azelastine and zileuton in patients with atopic dermatitis are at least partly mediated by the inhibition of the action and/or production of LTB_4 in the skin.

In conclusion, LTB_4 is increased in the skin and is involved in spontaneous itching in mice with chronic dermatitis. BLT1 receptor and 5-lipoxygenase in the skin may be effective pharmacological targets for the treatment of itch in atopic dermatitis.

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Labor, and Welfare Ministry, Japan; and the ONO Medical Research Foundation.

Author contributions

Dr Andoh (associated professor), Ms Haza (graduate student) and Ms Saito (undergraduate student) performed the experiments. They and Dr Kuraishi discussed research design and interpretation of data. Dr Andoh drafted the manuscript and Dr Kuraishi revised it critically.

Conflicts of interest

The authors state no conflict of interest.

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